J. Parasitol., 91(6), 2005, pp. 1280–1286

PARTIAL PURIFICATION AND CHARACTERIZATION OF AN AMINOPEPTIDASE FROM *EIMERIA TENELLA*

R. H. Fetterer, K. B. Miska, and R. C. Barfield

Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, United States Department of Agriculture, Henry A. Wallace Beltsville Agricultural Research Center, Beltsville, Maryland 20705. *e-mail: rfettere@anri.barc.usda.gov*

ABSTRACT: Our previous investigation demonstrated the expression in *Eimeria tenella* sporulated oocysts of an aminopeptidase (AP) with strong homology to AP N. To further understand the role of proteases during development, we investigated the molecular and biochemical properties of *E. tenella* AP. Greater than 95% AP activity was present in a soluble extract during sporulation of oocysts with highest activity in fully sporulated oocysts. The AP activity was inhibited by the AP inhibitors bestatin and 1,6-phenanthroline, but not by serine protease inhibitors. The AP had specificity for synthetic endopeptidase substrates that contain arginine, alanine, or glycine at the N terminus. Partial purification of the enzyme yielded a major protein band with an M_r of about 106 kDa and an isoelectric point (Ip) of 5.1. Reverse transcription-polymerase chain reaction indicated that the gene for AP is expressed during sporulation, but expression is absent or greatly reduced in the sporozoites and merozoites. On the basis of the deduced gene structure, the predicted M_r is 110 kDa with a pI of 5.59. Database search indicates that the *E. tenella* AP shares significant homology with the AP from Apicomplexan taxa: *Toxoplasma gondii*, *Cryptosporidium parvum*, and *Cryptosporidium hominis*. Together, these results confirm the presence of a cytosolic AP related to AP N, which is expressed and active during sporulation of *E. tenella* oocysts.

The malabsorption, reduced weight gain, and decreased efficiency of feed conversion resulting from the parasitism of the intestinal tract by several species of *Eimeria* causes significant economic losses to the poultry industry, which are estimated to be more than $800 million annually (Williams, 1998; Allen and Fetterer, 2002). Considerable interest is focused on the biology of the intracellular stages (sporozoites and merozoites, SZs and MZs, respectively) of the parasite because of their involvement with pathology (Allen and Fetterer, 2002) and their association with the host immune response (Jenkins, 1998; Lillehoj and Lillehoj, 2000). Less interest has focused on molecular and biochemical processes in the oocysts, the free-living infective stage of *Eimeria* spp.

Results of a recent characterization of genes differentially expressed during sporulation of *E. tenella* indicated an aminopeptidase (AP) with strong homology to AP N that may be developmentally regulated during sporulation (Miska et al., 2004). Aminopeptidase is a family of widely distributed exopeptidases that participate in many significant biological processes such as protein maturation, hormone production, and peptide digestion. Aminopeptidases have been demonstrated in several apicomplexan genera in which they have important functions in host cell invasion, immune responses, digestion, and excystment (Okhuysen et al., 1994; Nankya-Kitaka et al., 1998; Berthonneau et al., 2000; Padda et al., 2002). So far, characterization of AP from *Eimeria* spp. is limited. Multiple forms of AP from *E. tenella* unsporulated oocysts have been reported (Wang and Stotish, 1978) and leucine APs (LAPs) from *Eimeria falcimformis* was purified and characterized (Kaga et al., 1998). Due to the potential importance of APs in the regulation of biochemical processes during development, we report the partial purification and characterization of AP during sporulation of *E. tenella* oocysts.

MATERIALS AND METHODS

Host and parasites

Chickens (80–100 white leghorn–Rhode Island red sex-sals, Moyers Hatcheries Inc., Quakertown, Pennsylvania), 4–5 wk of age, were infected orally with 1.0–1.25 $\times$ 10^5 *E. tenella* (Wampler strain) oocysts per bird. On day 7 postinoculation (PI), birds were killed by cervical dislocation and the ceca were removed. Oocysts were recovered from infected ceca and sporulated as previously described (Fetterer and Barfield, 2003).

For studies of sporulation time-course, oocysts were suspended in phosphate-buffered saline (PBS) containing an antibiotic/antimycotic mixture (Gibco, Gaithersburg, Maryland) and incubated under aeration at 41 C. At the desired time interval (ranging from 0 to 72 hr), an aliquot containing about 1 by 10^8 oocysts was removed from the incubation flask, centrifuged, and the pelleted oocysts resuspended in 1.0 ml of 40 mM Tris and stored at −70 C.

Sporozoites were prepared from fully sporulated oocysts (less than 30 days postharvest) as previously described (Fetterer et al., 2004). Merozoites were collected from ceca at 108 hr PI from birds inoculated orally with 3 $\times$ 10^5 sporulated oocysts per bird. Merozoites were purified as described for SZ. Isolated SZ and MZ were resuspended in 40 mM Tris and frozen at −70 C.

Protein extract

Oocysts (1 by 10^8) suspended in 1 ml of 40 mM Tris or PBS were placed in 1.5-ml capped microfuge tubes containing 0.5 g of 0.5-mm glass beads and homogenized with a minibead-beater (BioSpec Products, Bartlesville, Oklahoma). Soluble extracts were prepared as previously described (Fetterer and Barfield, 2003). In some experiments, the buffer-insoluble material from oocysts was extracted with 0.2% t-octylphenoxypolyethoxyethanol (TX-100, Sigma St. Louis, Missouri) in 40 mM Tris pH 8.0 for 20 min at 4 C and centrifuged at 20,000 *g*, 4 C for 20 min and the supernatant retained as the TX-100 soluble fraction.

Sporozoites and MZs (3–5 by 10^8) were suspended in 0.5 ml of 40 mM Tris and homogenized with a sonic cell disruptor while maintained on ice. Homogenates were centrifuged at 104,000 *g* at 4 C and supernatants were retained as the soluble extract. Concentrations of soluble proteins were measured with a bicinchoninic acid assay (Pierce, Rockford, Illinois) with bovine serum albumin as the standard.

Enzyme assays

Aminopeptidase activity was measured using modifications of previously described methods (Rhoads and Fetterer, 1998) using synthetic peptidyl-7-amino-4-methyl coumarin (AMC) substrates unblocked at the N-termini (exopeptidase substrates). The AMC substrates (Bachem, Torrance, California) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 or 40 mM and diluted 10-fold in the assay buffer (AB; 50 mM Tris, pH 7.5) before addition to the assay. In some experiments, porcine LAP (E.C. 3.4.11.2, Sigma) was used as a positive control. The assay consisted of 170 μl of AB, 10 μl of enzyme preparation, and 20 μl of substrate in a 200-μl total volume. The enzyme inhibitors bestatin (BST) and 1,10-phenanthroline (PHEN) (Sigma) and

Received 30 December 2004; revised 16 March 2005; accepted 13 April 2005.

phenylmethylsulfonylfluoride (PMSF, Sigma) were dissolved in DMSO and diluted with AB, while 4-(2-aminoethyl)-benzesulfonyl fluoride (AEBSF, Roche, Mannheim, Germany) was dissolved in AB. Inhibitors, when used, were added in a 10-μl volume and allowed to incubate with enzyme for 10 min at room temperature before addition of substrate to start the reaction. Fluorescence was monitored (absorbance 380 nm, emission of 460 nm) in a microplate spectroflourimeter (Molecular Devices, Sunnyvale California). Assays were generally performed at 37 C for 10 or 15 min, while assays of chromatography fractions were conducted for 30 to 60 min. Enzyme activity was calculated as nanomoles of substrate per minute per milligram of protein.

Electrophoresis

Protein samples were analyzed by polyacrylamide gel electrophoresis (denatured and reduced) using 1-mm-thick gradient minigels (8 by 9 cm, 4–12% Bis Tris; Invitrogen, Carlsbad, California), fixed, and stained as previously described (Fetterer and Barfield, 2003). Nondenaturing substrate gels were performed using 12% Tris-glycine minigels (Invitrogen) with 25 mM Tris and 125 mM glycine as a running buffer. Samples were dissolved in sample buffer (100 mM Tris, 10% glycerol and 0.025% bromphenol blue) and separation was performed at 125 V for about 90 min. Gels were washed 3 times by incubation in 20 ml of AB for 20 min each then incubated for 40 min in AB containing 40 μM L-argAMC. Bands were visualized on a UV *trans*illuminator.

Protein fractions were separated by vertical gel isoelectrofocusing (IEF) using precast gels (pH 3–10; Invitrogen) as previoulsy described (Rhoads and Fetterer, 1998). Focusing was monitored with IEF standards (Invitrogen). Following focusing, gels were washed and bands were visualized for enzyme activity as described above. Gels were then fixed and stained with Coomassie blue (Simply Blue, Invitrogen) to visualize IEF standards.

Gels were calibrated for molecular weight or isoelectric point (Ip) by comparison with standards, and digitally photographed and analyzed with gel-imaging software (Labworks, UVP, Upland, California).

Enzyme purification

Soluble protein from sporulated oocysts (2 to 3 mg) in 40 mM Tris pH 8.0 was injected onto a high-performance liquid chromatography (HPLC) anion exchange column (DEAE 5PW, Protein-Pak, 7.5 by 75 mm steel, Waters, Milford, Massachusetts). The mobile phase consisted of 0.05 M Tris pH 8.0 (A) and 0.05 M Tris, 1.0 M NaCl, pH 8.0 (B). The flow rate was 1.0 ml per min at 22 C. The column was eluted with solvent A for 10 min, followed by a linear gradient from 100% A, 0% B to 50%A, and 50%B in 35 min. Aliquots were collected at 0.5-min intervals (0.5 ml) and absorbency was monitored with a photodiode array detector (PDA, Waters Model 996). The 0.5-ml aliquots were pooled on the basis of AP activity. After dialysis against PBS and concentration to about 300 μl, the fraction enriched for the AP activity was further purified by size exclusion (SEC) HPLC. The sample was applied to G-250 (Phenomenex, Torrance, California) and I-125 (Waters) in SEC columns in series. The columns were eluted with 50 mM KH_2PO_4 containing 0.15 M NaCl pH 7.0, for 30 min at 22 C, with a flow rate of 1 ml/min and absorbance monitored with a PDA detector. Aliquots (0.5 ml) were collected, dried under vacuum, resuspended in sample buffer, and analyzed by polyacrylamide gel electrophoresis and by silver staining.

Sequence and evolutionary analyses

To identify the structure of AP in the genome of *E. tenella*, a 246 base pair (bp) gene fragment, which was identified through random screening of an *E. tenella* subtracted cDNA library constructed from sporulated oocysts (Miska et al., 2004) (GenBank accession number CF983608), was used to search the *E. tenella* genome using the OmniBlast server located at the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/e_tenella/omni). In an effort to determine whether AP homologues exist in other Apicomplexans, we searched the protist enhanced sequence tag (EST) databases maintained by The Institute for Genomic Research (TIGR) (http://www.tigr.org/tdb/tgi/protist.shtml) using the nucleotide Basic Local Alignment Search Tool (BLASTN) (Altschul et al., 1990). Amino acid alignments were constructed using the ClustalX software (Thompson et al., 1997) with minor manual corrections. Conserved domains for peptidases were identified using SMART (a Simple Modular Architecture Research Tool), which allows the identification and annotation of genetically mobile domains and the analysis of domain architectures (Schultz et al., 2000).

Developmental expression of the AP gene

All tissues used in RNA isolation were snap-frozen following purification and were stored at −70 C until use. Total RNA was isolated from MZs, SZs, sporulated oocysts, and unsporulated oocysts of *E. tenella* using TRIzol (Invitrogen). Each sample was combined with approximately 3 g of diethylpyrocarbonate (DEPC)-treated Pyrex beads (3-mm diameter) (Corning, New York) and 10 ml of TRIzol. The sample was vortexed for 1 min, then incubated on ice for 1 min (4×). The remainder of the total RNA isolation protocol was carried out using the manufacturer's recommended instructions. The resulting pellets containing total RNA were resuspended in DNase/RNase-free water (Invitrogen) and were stored until cDNA synthesis at −70 C. Before cDNA synthesis, a portion of the *E. tenella* total RNA was treated with DNase I (Invitrogen) using the manufacturer's instructions. Complementary DNA was synthesized from 0.8 μg of total RNA using the recommended instructions provided with the Advantage RT for PCR kit (BD Biosciences Clontech, Palo Alto, California) using oligo dT or random hexamer primers. Aminopeptidase transcripts were amplified from 5.0 μl of cDNA with Taq DNA polymerase (CLP, San Diego, California) in the presence of 10 μM primer. Forward primer KM132b located in exon 2 (5′-TCGATGACGCTAACACGAAGG-3′) was used in combination with reverse primer KM133b, located in exon 6 (5′-AGCAAGAACACGCTCGAAAT-3′) to amplify the transcript from *E. tenella* cDNA. The amplifications were carried out as follows: denaturation, 94 C for 30 sec; annealing, 65 C for 30 sec; extension, 72 C for 1 min; and a final extension of 5 min at 72 C. Cycles 2–4 were repeated 30 times.

RESULTS

Enzyme activity

Aminopeptidase activity as measured by hydrolysis of the synthetic substrate L-argAMC was detected in soluble extracts of all the *E. tenella* developmental stages examined (Fig. 1A). The AP activity was highest in the extracts from the 72-hr sporulated oocysts and declined in the SZ and MZ stages. In the unsporulated and sporulated stages, 95% and 99% respectively, of the AP activity was detected in the soluble fraction relative to the TX-100 extracted fraction (data not shown). The AP activity was significantly inhibited by both PHEN and BST with the concentration required to inhibit AP activity by 50% was about 100 μM for BST and 750 μM for PHEN (Fig. 1B). The serine protease inhibitors AEBSF and PMSF were without effect at a concentration of 1,000 μM (data not shown).

Substrate specificity of the soluble AP from sporulated oocysts relative to hydrolysis of L-arg AMC (1.0) indicated L-glyAMC and L-alaAMC were hydrolyzed at a very similar rate to that of L-arg (1.04); followed by L-LysAMC (0.7) and L-LeuAMC (0.32); and L-GlucAMC, L-ProAMC, and L-PhenAMC (< 0.1).

Substrate gels

Substrate gel electrophoresis was performed to determine whether multiple forms of the AP are expressed during development. Nondenaturing gel electrophoresis of oocyst-soluble proteins after 0 to 72 hr of sporulation indicate a primary, single band of enzyme activity after incubation with L-argAMC as a substrate. Very minor bands with higher mobility were observed for some of the time points (Fig. 2A). Porcine LAP had a lower mobility than that of AP activity from oocysts. Isoelectric focusing substrate gels indicate that soluble extracts

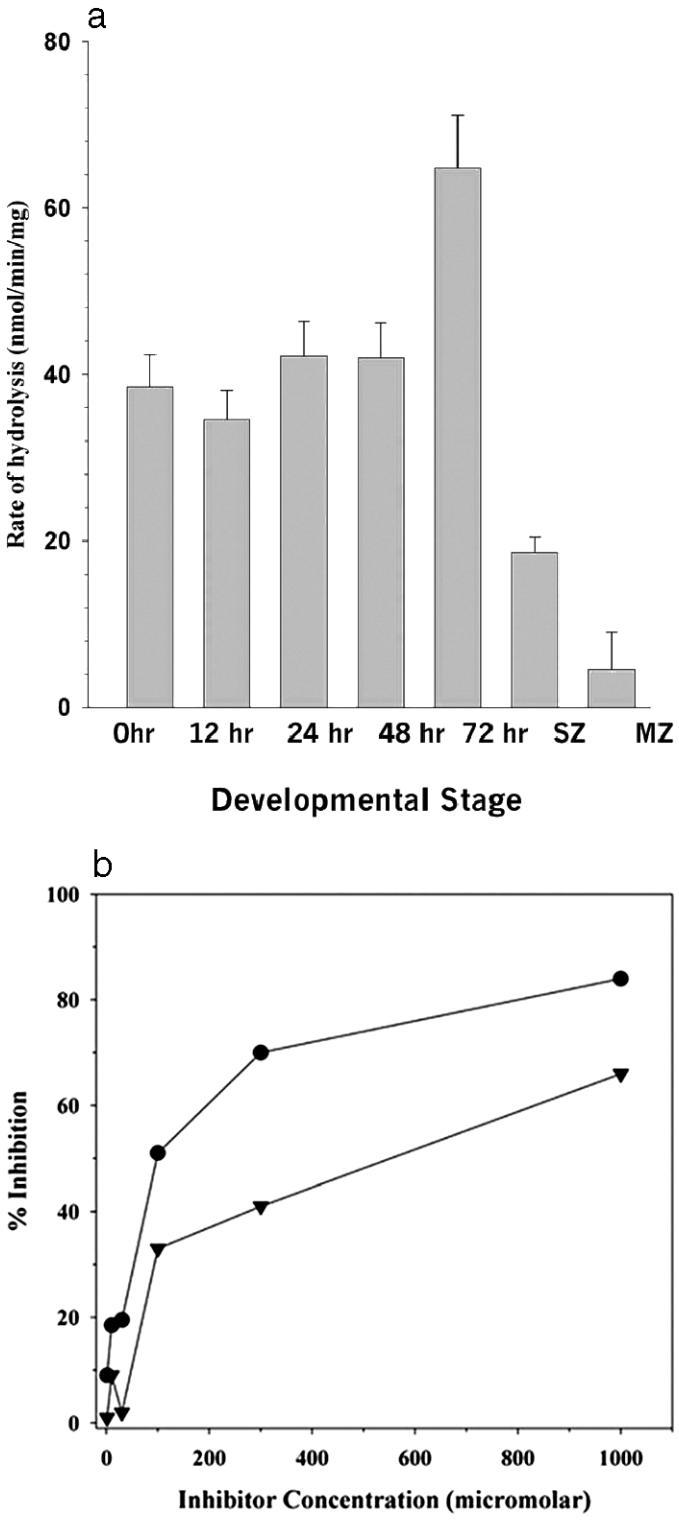

FIGURE 1. Aminopeptidase (AP) activity of soluble extracts from *E. tenella*. **A.** Aminopeptidase activity in oocysts from 0 to 72 hr of sporulation and sporozoites (SZ) or merozoites (MZ). The assay was performed using L-argAMC as substrate. Assays were incubated for 15 min at 37 C. Values are means of 3 measurements with vertical bars representing 1 SD. **B.** Effect of the AP inhibitors bestatin (circles) and phenantholine (triangles) on hydrolysis of L-argAMC by soluble extracts of *E. tenella* sporulated. The inhibitors were incubated for 5 min with extract before addition of L-argAMC to start the reaction and then incubated for 15 min at 37 C. Values are means of 3 replicates.

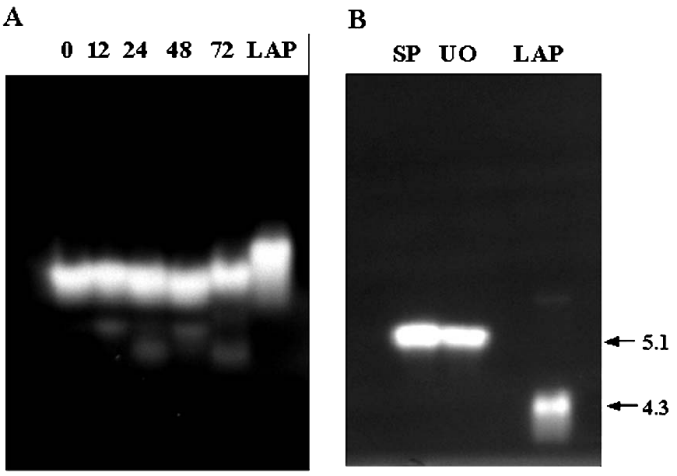

FIGURE 2. Substrate gel electrophoresis of soluble extracts from *E. tenella* oocysts. **A.** Nondenaturing gel electrophoresis of *E. tenella* sporulated oocysts from 0 to 72 hr of sporulation. Porcine leucine aminopeptidase (LAP) was included as a positive control. **B.** Isoelectric focusing (IEF) substrate gels of unsporulated (UO) and sporulated (SP) oocysts. Arrows indicate calculated Ip for extracts from oocysts and LAP.

from unsporulated and sporulated oocysts also had single bands of activity corresponding to an Ip of 5.1, whereas LAP had an Ip of 4.3 (Fig. 2B).

Purification

Aminopeptidase in the soluble fraction of sporulated oocysts was enriched about 10.5-fold (28% yield) after initial fractionation by HPLC-AX chromatography and 220-fold (2% yield) by second-step HPLC-SEC chromatography (Fig. 3A). Electrophoretic analysis of the purified fraction indicates a prominent protein of 105 kDa with a less abundant band of 82 kDa (Fig. 3B).

Developmental expression of AP during *E. tenella* life cycle

Aminopeptidase transcripts were amplified from *E. tenella* cDNA synthesized from oocysts at different sporulation time points, as well as SZs, and MZs. The primer pairs used in amplification were designed to span an intron; therefore, polymerase chain reaction (PCR) products generated from cDNA were smaller than intron-containing products amplified from genomic DNA (Fig. 4). The expected size of products amplified from cDNA was 910 base pairs (bp), while those amplified from genomic DNA were 1,672 bp in length. The reverse transcription-PCR results are shown in Figure 4, and it is clear that AP is expressed during sporulation. However, it appears that as sporulation progresses, transcript levels are decreased, with little or no detectable AP expression observed in SZs and MZs.

Genomic organization cDNA structure of *E. tenella* AP

The *E. tenella* AP was initially located by performing a BLAST search using a partial-length cDNA isolated from *E. tenella* sporulated oocysts to search the sequence of the *E. tenella* genome. A single match was found in a segment in contig 12747 that consists of 412,739 nucleotides of *E. tenella* DNA.

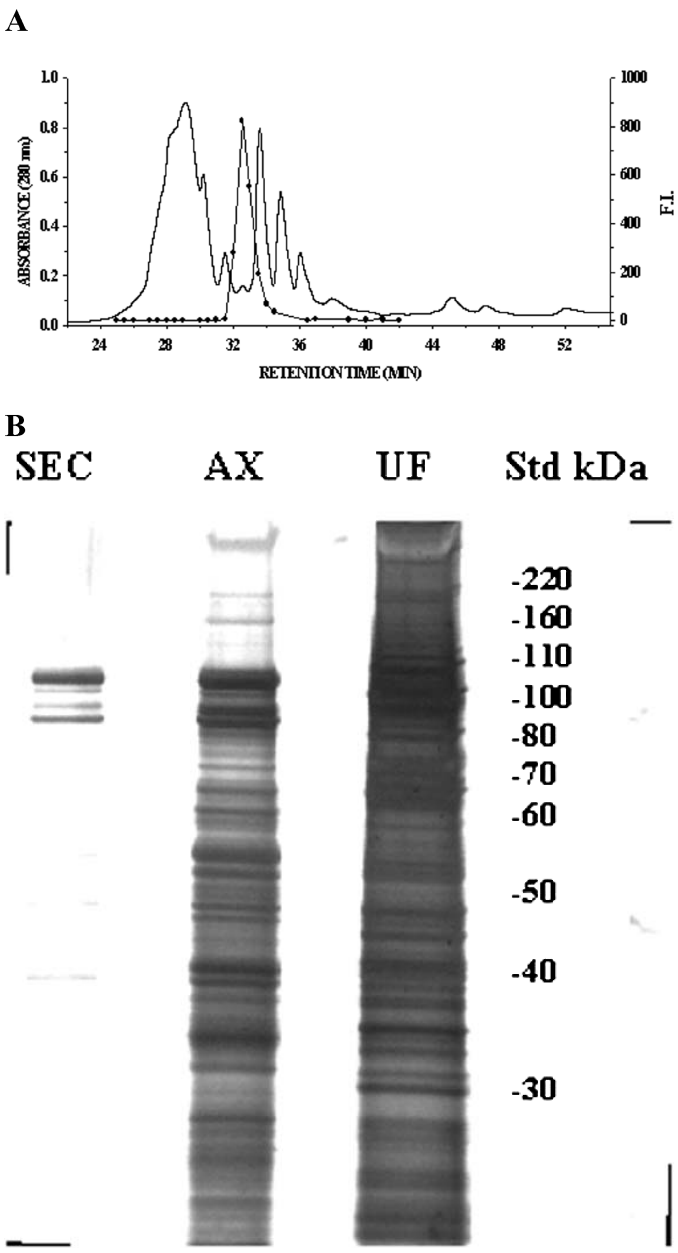

FIGURE 3. Purification of AP activity from *E. tenella* sporulated oocysts. **A.** Anion exchange HPLC separation of proteins (absorbance 280 nm, solid line) and activity with L-argAMC as substrate AP activity (fluorescence intensity, F.I.; circles). Fractions containing AP activity were pooled and applied to an HPLC-size exclusion column (inset). Fractions containing AP activity (circles) were pooled and analyzed by gel electrophoresis. **B.** Silver-stained gels containing unfractionated starting material (UF), anion exchange (AX), and size exclusion (SEC) separations.

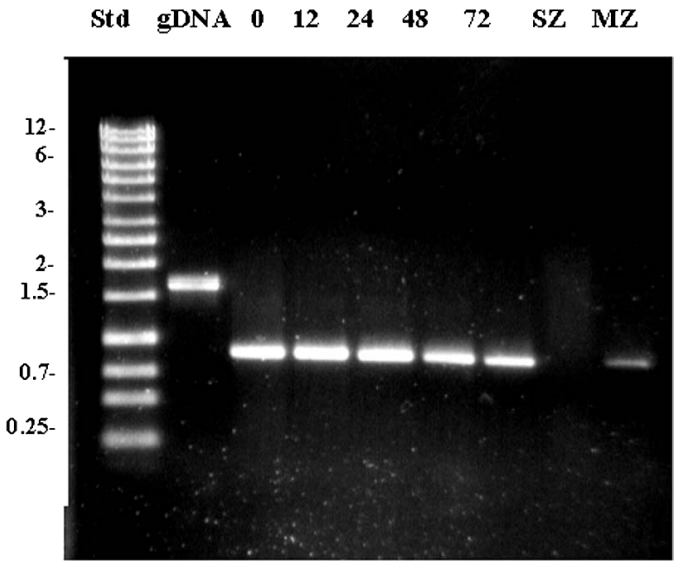

FIGURE 4. Expression of aminopeptidase in developmental stages of *E. tenella*. Transcripts were amplified from cDNA synthesized from oocysts at different sporulation time points (0–72 hr), as well as from sporozoites (SZ), and merozoites (MZ). The expected products amplified from cDNA were 910 bp in length, whereas those amplified from genomic DNA (gDNA) were 1,672 bp in length. Standards are given in kilobase pairs.

Eimeria tenella AP most likely represents a single-copy gene because only a single significant (E-value < 1 by 10^{-20}) match was found during the genome search. To determine the putative intron/exon boundaries, the sequence likely to contain AP was compared with known APs. Additionally, exons 2–7 and 9–11 were confirmed by obtaining cDNA sequence. The deduced structure of *E. tenella* AP is shown in Figure 5. The gene consists of 13 exons and 12 introns. The open reading frame spans 2,772 bp, encoding 923 amino acids. On the basis of this sequence, the predicted molecular weight of *E. tenella* AP is approximately 104 kDa, with a pI of 5.59. The putative amino acid sequence is shown in an alignment (Fig. 6). By searching the protist EST database maintained by TIGR we were able to identify tentative contigs (TCs) composed of overlapping ESTs that share significant homology to *E. tenella* AP from several apicomplexan taxa (i.e., *Toxoplasma gondii*, *Cryptosporidium parvum*, and *Cryptosporidium hominis*. The sequences from these 3 species encode a full-length AP, and the amino acid translations are shown aligned in Figure 6. Because plant APs also show sequence homology to the protozoan gene the sequence of *Arabadopsis thaliana*, AP N is also included for comparison.

DISCUSSION

A previous study indicated the expression of an AP gene in *E. tenella* sporulated oocysts (Miska et al., 2004). The present result confirms the presence of an AP in the oocyst that appears to be closely related to AP N. Consistent with properties of an AP, the hydrolysis of synthetic substrates by oocyst extracts was inhibited by both the AP inhibitor BST and the metal chelator PHEN, but not by the serine proteases inhibitors AEBSF and PMSF. Hydrolysis of exopeptidase substrates was observed in oocyst extracts, but levels of enzyme activity were about 10-fold less than that of AP and were inhibited by serine protease inhibitors, but not by BST or PHEN (R. Fetterer, unpubl. obs.), indicating that the hydrolysis of the endopetidase substrates is due to AP. The AP displayed substrate preference for N-terminal arginine and alanine typical of AP N, and leucine was hydrolyzed at a 70% lower rate, suggesting the enzyme is not a leucine AP. However, a phenylalanine substrate was not significantly hydrolyzed, differing from the substrate specificity of

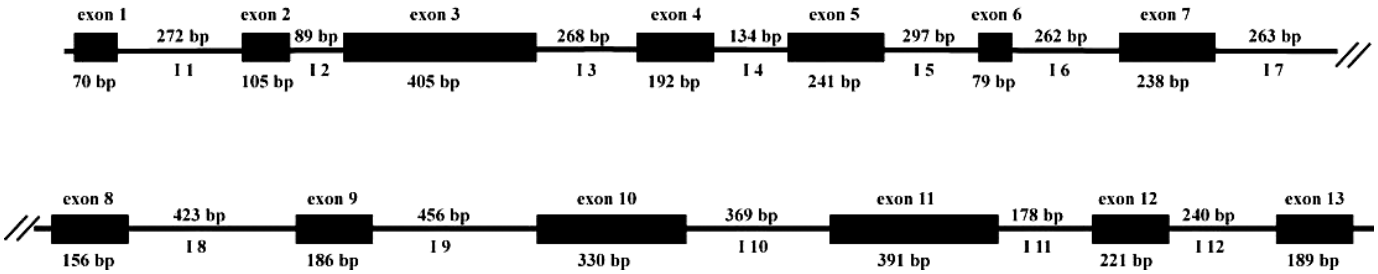

FIGURE 5. Intron/exon organization of *E. tenella* aminopeptidase.

an AP N from *C. parvum* (Okhuysen et al., 1994). Preference for glycine at the N terminus is characteristic of glycyl APs found primarily in bacteria (Rawlings and Barrett, 1995) and is, interestingly, consistent with a conserved domain for glycyl AP observed in the AP gene structure.

The *E. tenella* AP gene has a high degree of homology to AP N, primarily from plant and bacterial origin. The highest homology was observed with *A. thialiana* AP N. There is also significant homology with apicomplexan AP, including AP N from *C. parvum*, *C. hominis*, and *T. gondii*. From the amino acid alignment, it is also clear that APs are highly diverse proteins, with the N and C termini exhibiting high heterogeneity, while the middle parts of the protein (approximately from 200 to 530 amino acids) show a higher degree of conservation and contains conserved domains for AP and glycyl AP. The putative zinc ion ligand, the glutamyl residue involved in catalysis, and the conserved putative proton donor are all present in the sequence consistent with a function as a metalloexopeptidase. The

```
                . . . .10 . . . .20 . . . .30 . . . .40 . . . .50 . . . .60 . . . .70 . . . .80 . . . .90 . . . 100 . . . 110 . . . 120
E. tenella  .MSMEPSTLFFLCSPT.....................SRWPQRRGRPGEKFRDEYKPSDFTVESVDLNFLLDDANTKVSSDDANTKVFATIILKR.AEGTVPTDLVLNG.EDMELLSLKV
T. gondii   MVTLSAT-MNATV--CRISGGVSSRLCGWGVLSFLLA-VITVQVS-A---.EK-EPKAVVRLDYKPP--Y-E-IVLDFNL--ED-T-E-LLT-Y-.RA--E-MN---D-D-SVI-K-ISL
C. parvum   ........MGN-FGKCG.....................CRDLTICNLNVLRPAHSQDRIYRK-YKVP---I-HV-LDINIK-DV-V-SSV--MR-NPNSSFRG--S-D-.DCLK-V-V-L
C. hominis  ........MGN-FGKCG.....................CRDLTICNLNVLRPAHSQDRIYRK-YKVP---I-HV-LDINIK-DV-V-SSV--MR-NPNSSFRG--S-D-.DCLK-V-V-L
A. thaliana .....................................................MDAPKEI-LKNYTKPDYYFETVDLSF-LGEEK-I-SSK-KVSPRVK-SS.AA---D-.H-LK---V--

                . . . 130 . . . 140 . . . 150 . . . 160 . . . 170 . . . 180 . . . 190 . . . 200 . . . 210 . . . 220 . . . 230 . . . 240
E. tenella  NGKVVEELK.ENPKGGDEGAAKQGGKEGYRLGSDD.VLIVTKEALPQDAGETFTLQTEVSIHPVTNLKLKGLYVSGTALVTQCEAEGFRRITYFLDRPDVMATYKVRLEANKAKYPILLS
T. gondii   ATTSSPS-RGQGRVTFT-LDGQMPTDSQFQFRIV-GN-M-S-FL--RE-EVR-YIR-L---N-KA--A-F---KA-DIF--LN-PS-------GV-----L---T-TVT-PR.HL-----
C. parvum   --VIL-KSL.............Y-GYFQPDGP-GK-VISSNL--N.KDQQ---E-V-E-F-DR-T-NM---Y-AGVYS----SD------F--------CKFR--I-GD-T-S-V---
C. hominis  --IIL-KSL.............Y-GYFQPDGP-GK-VISSNL--N.KDQQ---E-V-E-F-DK-T-NM---Y-AGVYS----SD------F--------CKFR--I-GD-T-S-V---
A. thaliana E--LLK-.................GD-Q-D-R..H-TLPS..----E-S-V-EIDTE-Y-HK-TS-E---K-SGNFC---------K--FYQ----I--K-TC-V-GD-TL--V---

                . . . 250 . . . 260 . . . 270 . . . 280 . . . 290 . . . 300 . . . 310 . . . 320 . . . 330 . . . 340 . . . 350 . . . 360
E. tenella  NGNKADEGPVEDAPHRHFAVFDDPHPKPSYLFAILAGNFEAIRDTFTTKSGKDVALAVYSEPAQLHKLNWAMHSVKVSMKWEEDNFGREYDLDAFNVACVSDFNAGAMENKGLNIFNCTL
T. gondii   --D-VLS-..YAGEN----K-V--F--------LV--D-ASVSGE-V-M--RS-TVTI-AQHH-RNQ-Q--LR-LLRA-R-D-ET-----QYSE-R-L--EV--P-----TS----T-S-
C. parvum   ---LLEK-D-QGSEN----I-V--F---C----VV--VLGRLE-K-I-----T-R-F-----KYVDR-RL--E-L-LA---D--R--L----EI--IVA-ES--F------S------SC
C. hominis  ---LLEK-D-QGSEN----I-V--F---C----VV--VLGRLE-K-I-----T-R-F-----KYVDR-RL--E-L-LA---D--R--L----EI--IVA-ES--F------S------SC
A. thaliana ---LISQ-DI-GG..--Y-LWE--PK--C----LV--QLVSRD-----R--RQ-S-KIWTPAED-P-TAH--Y-L-AA---D--V--L-----L--IVA-P---M------S-----SK-

                . . . 370 . . . 380 . . . 390 . . . 400 . . . 410 . . . 420 . . . 430 . . . 440 . . . 450 . . . 460 . . . 470 . . . 480
E. tenella  LLASMDTSTDEDFERVLAIIGHEYFHNWTGNRVTVRDWFQLTLKEGLTVFREQLFMGSVLSAGVQRIQEVADLISRQFAEDDGPMAHPIRPESYLAMDNFYTATVYDKGAEVVRMYHTLL
T. gondii   ---DPKLT--A-HRLIVDVVS-------------Q------------M--NNS-TEETT-RAMK--GD---IL-I--R--S--F-------T-KSIE-L--R---L-----N---R-M-
C. parvum   ----ENITP-YF-TNI-S-V-------Y------C-------------Y-D-E-SRDCIDRLSEQLGDIEV-RNY--Q--S--L------D-IVSTN-L--S---R---------E-I-
C. hominis  ----ENITP-YF-TNI-STV-------Y------C-------------Y-D-E-SRDCIDRLSEQLGDIEV-RNY--Q--S--L------D-IVSTN-L--S---R---------E-I-
A. thaliana V---PE-A--A-YAAI-GV--------------C------S---------D-E-SSDMG-RT-K--AD-SK-RIY--PQ-A------V--H--IK------V---E---------K---

                . . . 490 . . . 500 . . . 510 . . . 520 . . . 530 . . . 540 . . . 550 . . . 560 . . . 570 . . . 580 . . . 590 . . . 600
E. tenella  GTPGFRKGMDLYFQRHDNQAATCDDFRAAMADSNGVNLDQMERWYTQAGTPRLEVLKAALNKETKVFEIRFKQYTPATPGQNKKLPQVIPIKMGLIGKTS.............RRELVQP.
T. gondii   -PK---R------K---GK-V----L------A-DKD-S-F----S-----HVT-SSFIYDAAERKMHLTL--H--P----D----LQ--VAV-C-----.............K-DVLS-P
C. parvum   -RE----------A---G--V------K--E-A-NY-FT-F----D-----EV--VSIDH--AEGTCS-TLR-RCSP--E-P--R-FY--VIV-----D.............SCKEIRQS
C. hominis  -RE-----I----A---G--V------K--E-A-NY-FT-F----D-----EV--VSIDH--AEGTCS-TLR-RCSP--E-P--R-FY--VIV-----D.............SCKEIRQS
A. thaliana --Q-----I----E---E--V--E--F---R-A-NADFANFLQ--S-----VVK-VSSYNADAR.T-SLK-S-EI-P----PT-E-TF--VVV--LDSSGKDITLSSVHHDGTVQTISGS

                . . . 610 . . . 620 . . . 630 . . . 640 . . . 650 . . . 660 . . . 670 . . . 680 . . . 690 . . . 700 . . . 710 . . . 720
E. tenella  AVVLEMTEEEQTFRLNNISEDCVPSILRGFSAPVI.LVNPHQTEEDMAFLMAYDSDPVTKWFASRALATPIILSRASQVVANKNVR.........IFEQISGAYIDALR..TTLTDNTLD
T. gondii   TQI--L-----A-V-VDVG----L-V--D-----K.-IQ-L--D--L---VT----N-NR-Q-AQT-SRKVL-T-I-EYQQRVEEIEEGRVSESDL-SKLPTQ-VQTV-DIVIAPESSMG
C. parvum   ETLI.LK-QQ---I-DGVW-TP-A----------NLRFKTPRSD-EL---F-F-T-EYNRFD-AQT-YKK-LIQASTNSSQEISPSSVIESILSQTILSCFESF-SKIKT.SKNPNG-VS
C. hominis  ETLI.LK-QQ---I-DGVW-TP-A----------NLRFKTPRSD-EL---F-F-T-EYNRFD-AQT-YKK-LIQASTNSSQEISPSSVIESILSQTILSCFESF-SKIKT.SKNPNG-VS
A. thaliana STI-RV-KK-EE-VFSD-P-RP---LF--------..R-ETDLSND-LF--L-H---EFNR-E-GQV--RKLM-NLV-DFQQ--P......LALNPK-V-GL-SVLS.........-SS--

                . . . 730 . . . 740 . . . 750 . . . 760 . . . 770 . . . 780 . . . 790 . . . 800 . . . 810 . . . 820 . . . 830 . . . 840
E. tenella  NALKALLLQLPDWSTLSTHMKTIDPEALHLAIRSVKADVAAALKTEMAKEYEHLTLPAG.EEDDME........EESTGRRKLRNTLLFFLSESRDREAVDRAVKHFTDAKCMTDRYAGL
T. gondii   KDI-S---S--TKAQ-ELAVDS---D-INA-LA--RR-IVD--GE--LQL-TE------T--SGAD........I-HW---A---E--R--TA-F-QKSAKL-SA--DR-MV-S-KV-A-
C. parvum   PMVASYT-RI-SY-QVLASIAEPNFDFVVESFNALTVAF-SRF--QIVDLFQV--EDLSALPSQSPFIHGKGVNV-AIAI-R-L-I--D-IAKLDPSLGTKL-FEQHNRFDY--SKLGAI
C. hominis  PMVASYT-RI-SY-QVLASIAEPNFDFVVESFNALTIAF-SKF--QIVDLFQV--EDLSALPSQSPFIHGKGVNV-AIAI-R-L-I--D-IAKLDPSLGTKL-FEQHNRFDY--SKLGAI
A. thaliana KEFI-KAIT--GEGEIMDM-AVA--D-V-AVRKF-RKQL-SE--E-LL-IV-NNRSTEAYVF-HS...........NMA--A-K--A-AY-ASLE-PAYMEL-LNEYKM-TNL--QF-A-

                . . . 850 . . . 860 . . . 870 . . . 880 . . . 890 . . . 900 . . . 910 . . . 920 . . . 930 . . . 940 . . . 950 . . . 960
E. tenella  ITLGNIPTAERE..TAFARFYEDAAGDPLVLDKWFRAQARSDLPDQVERVAELLN.HPAFSLK..NPNRLRSLIFTFASLNPLHFHRSDGKGYELLANVILEVDRINPVAASRGA.KLFL
T. gondii   TV-TE--GQ--D..E--E--RQEV--NAVLME--MSL--M-AAA-TL----Q-RQ.-S--HPF..I-GSAY--FG--S-SS-.Q--SKT-A--A-VLDFLMMI-EH-SLVS--I-GVA-A
C. parvum   SA-QIADHKSS-CIQ-LDSLLLATSD-VST-NQY-SI--SCLI--N----INIYHSN-Q-IKYRE--TIFS--VG----.-FVA-N-K--L--SFV-DA-ILM-KV--MS-ASVS.RA-T
C. hominis  SA-QIADHKSS-CIQ-LDSLLLATSD-VST-NQY-SI--SCLI--N----INIYYSN-Q-IKHRE--TIFS--VG----.-FVA-N-K--L--SFV-DA-ILM-KV--MS-ASVS.RA-T
A. thaliana AA-SQN-GKT-D..DIL-D--NKWQD-Y--VN---LL-ST--I-GN--N-KK--D.----D-R..---KVY---GG-CG.S-VN--AK--S--KF-GDIVVQL-KL--QV---MV.SA-S

                . . . 970 . . . 980 . . . 990 . . .1000 . .
E. tenella  QWRRYNAHRQRLIESQLHRILSAKTLSKNVKEVVSMALDSPHSSP
T. gondii   N-K-FTSP-REMLKEV-KKL-KKEK--VGL---LEK--AGEDD..
C. parvum   KVLKLDEG-RN-LKENVV---QSPG---DTS-IL.KSI-F.....
C. hominis  KVLKLDEG-RN-LKENVV---QSPG---DTS-IL.NSI-F.....
A. thaliana R-K--DET--G-AKA--EM-M--NG--E--F-IA-KS-AA.....
```

FIGURE 6. Amino acid alignment of aminopeptidase from 4 species of protozoa (*E. tenella*, *T. gondii*, *C. parvum*, and *C. hominis*), and a single plant species, *A. thaliana*. Dashes indicate sequence identity and dots indicate gaps in sequence. The position of the conserved zinc ion ligand (L), both the conserved residue involved in catalytic site (C) and conserved proton donor (D), are indicated in bold on top of the sequence.

molecular analysis of the deduced gene structure indicates that the *E. tenella* AP is present as a single-copy gene. This result corroborates findings in *C. parvum*, which also indicate that in this species AP exists as a single-copy gene. Additionally, the *E. tenella* AP appears to be a single molecular species as indicated by the presence of single bands by native gel electrophoresis and isolectric focusing. These findings are in contrast to an older study that indicated the presence of 3 LAP isozymes during sporulation (Wang and Stotish, 1978). The reasons for the discrepancies are unclear, but because the previous authors used LAP substrates, they may be detecting a different enzyme than reported in the present study. The *E. tenella* AP gene sequence lacks transmembrane segments and little AP activity is associated with the detergent-extracted fraction, suggesting the enzyme is cyctosolic. The AP from *T. gondii* tachyzoite (TZ) is also predominantly soluble (Berthonneau et al., 2000). However, the AP N gene from *C. parvum* also lacks transmembrane domains, but the enzyme is found nearly exclusively in the detergent-soluble fraction and is associated with the SZ surface membrane (Padda et al., 2002), suggesting that the transmembrane segments may not be predictive for cellular location of this enzyme. Studies to further localize the *E. tenella* AP are in progress and will aid in determining more precisely the cellular location of the enzyme.

Although purification to homogeneity was not obtained in the current study, a fraction highly enriched in AP was obtained containing a major band with an M_r of 106 kDa, which is quite close to the M_r predicted from the gene sequence (110 kDa). The M_r observed for *E. tenella* AP is larger than that observed for *C. parvum* (89 kDa), and is identical to the M_r of 110 kDa reported for *T. gondii* AP (Berthonneau et al., 2000). In addition, the experimentally determined Ip of the AP (5.1) is similar to that predicted from the gene sequence (5.8). The M_r and Ip for am AP from *Eimeria falciformis* that hydrolyzed a leucine substrate from sporulated oocysts were 50 and 8.6 kDa, respectively (Kaga et al., 1998). The differences in M_r and Ip from those for *E. tenella* suggest that the *E. falciformis* AP is a different enzyme, possibly LAP, than that of the AP reported in the present study.

The function of AP during *E. tenella* sporulation is speculative, but the enzyme is probably involved in the extensive protein processing that most likely occurs during the rapid development of the oocysts. Aminopeptidase was detected at all time points examined during the course of *E. tenella* sporulation with lesser activity in the SZ and MZ stages, supporting a conclusion that the AP appears to be primarily associated with sporulation. Expression of the AP gene during sporulation was observed, but little if any expression was noted in the SZ or MZ stages. Although only qualitative in nature, this observation also suggests a diminished role for AP in the intracellular stages. This is in contrast to an observation of the AP from other Apicomplexa that suggest a significant role for AP in the intracellular stages. Significant levels of AP are observed in *T. gondii* tachyzoites and AP inhibitors reduce cell invasion and growth in vitro. *Cryptosporidium parvum* sporulated oocysts contain an AP that is associated with excystment and may be located on sporozoite surface membranes (Okhuysen et al., 1994). Aminopeptidases in *Plasmodium chaubaudi* and *Plasmodium falciparum* are associated with terminal digestion of hemoglobin by the intracellular stages, and AP inhibitors also reduce growth and development of the parasite (Nankya-Kitaka, 1998). Although the expression and enzyme levels of AP are reduced in *E. tenella* SZs and MZs, it is possible that AP or a related enzyme plays an important function in these extracelluar stages. Studies are in progress to examine the effect of AP inhibitors on cell invasion and intracellular growth.

Knowledge of the occurrence and function of proteolytic enzymes in development of *E. tenella* is minimal. Of the 4 major catalytic classes of peptidases, only aspartic proteases (eimepsin) are developmentally regulated (Jean et al., 2001). Limited evidence suggests that serine proteases are present in the sporulated oocysts and may mediate cellular invasion by SZs (Fuller and McDougald, 1990; Milkaski et al., 1994). Consistent with these findings, a serine protease related to subtilisin has been shown to be expressed in the sporulated oocysts (Miska et al., 2004), and a number of enzymes with serine protease activity have been observed in both unsporulated and sporulated oocysts (R. Fetterer, unpubl. obs.). Cysteine proteases have not been reported from *E. tenella*, although homologs to known cysteine proteases can be found in the *E. tenella* genome. Our current work documents that a proteolytic enzyme of the metalloprotease class related to AP N is present during development of oocysts and most likely function in protein processing, but its precise role as well as the more general role of proteases during development, has yet to be determined.

ACKNOWLEDGMENTS

The authors are grateful for the expert technical assistance of Gary Wilkins, Jenifer Herrmann, Mark Paape, and Eli Miramontes. We are also indebted to M. Shirley for granting permission to use the *E. tenella* genome sequence available at the Sanger Institute Web site. USDA/ARS CRIS project 1265-31320-070-00D supported this work.

LITERATURE CITED

ALLEN, P. C., AND R. H. FETTERER. 2002. Recent advances in biology and immunobiology of *Eimeria* species and the diagnosis and control of infection with these coccidia parasites of poultry. Clinical Microbiological Reviews **15:** 58–65.

ALTCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS, AND D. J. LIPMAN. 1990. Basic local alignment search tool. Journal of Molecular Biology **215:** 403–410.

BERTHONNEAU, J., M.-H. RODIER, B. E. MOUDINI, AND J.-L. JACQUEMIN. 2000. *Toxoplasma gondii*: Purification and characterization of an immunogenic metallopeptidase. Experimental Parasitology **95:** 158–162.

FETTERER, R. H., AND R. C. BARFIELD. 2003. Characterization of a developmentally regulated oocyst protein from *Eimeria tenella*. Journal of Parasitology **89:** 553–564.

———, K. B. MISKA, M. C. JENKINS, AND R. C. BARFIELD. 2004. A conserved 19 kDa *Eimeria tenella* antigen is a profilin-like antigen. Journal of Parasitology **90:** 1321–1328.

FULLER, A. L., AND L. R. MCDOUGALD. 1990. Reduction in cell entry of *Eimeria tenella* (Coccidia) sporozoites by protease inhibitors and partial characterization of proteolytic activity associated with intact sporozoites and merozoites. Journal of Parasitology **76:** 464–467.

JEAN, L., P. DUNN, P. BUMSTEAD, K. BILLINGTON, R. RYAN, AND F. TOMLY. 2001. Genomic organization and developmentally regulated expression of an apicomplexan aspartyl proteinase. Gene **262:** 129–136.

JENKINS, M. C. 1998. Progress on developing a recombinant coccidiosis vaccine. International Journal for Parasitology **28:** 1111–1119.

KAGA, M. M., F. LAURENT, A. DOH, G. LUFFAU, P. YVORE, AND P. PERY. 1998. Purification of a leucine aminopeptidase for *Eimeria falciformis*. Veterinary Research **29:** 107–111.

LILLEHOJ, H. S., AND E. P. LILLEHOJ. 2000. Avian Coccidiosis. A review

of acquired intestinal immunity and vaccination strategies. Avian Diseases **44:** 408–425.

MILKASKI, W. P., J. K. CROOKS, AND S. L. PROWSE. 1994. Purification and characterization of a serine-type protease from *Eimeria tenella* oocysts. International Journal for Parasitology **24:** 189–195.

MISKA, K. B, R. H. FETTERER, AND R. C. BARFIELD. 2004. Analysis of transcripts expressed by *Eimeria tenella* oocysts using subtractive hybridization. Journal of Parasitology **90:** 1245–1252.

NANKYA-NKITAKA, M. F., G. P. CURLEY, C. S. GAVIGAN, A. BELL, AND J. P. DALTON. 1998. *Plasmodium chabaudi chabaudi* and *P. falciparum;* inhibition of aminopeptidase and parasite growth by betstatin and nitrobestatin. Parasitology Research **84:** 552–558.

OKHUYSEN, P. C., H. L. DUPONT, C. R. STERLING, AND C. L. CHAPPELL. 1994. Arginine aminopeptidase, an integral membrane protein of the *Cryptosporidium parvum* sporozoite. Infection and Immunity **62:** 4467–4670.

PADA, R. S., A. TSAI, C. L. CHAPPELL, AND P. C. OKHUYSEN. 2002. Molecular cloning and analysis of the *Cryptospordium parvum* aminopeptidase N gene. International Journal for Parasitology **32:** 187–197.

RAWLINGS, N. D., AND A. J. BARRETT. 1995. Evolutionary families of aminopeptidases. Methods in Enzymology **248:** 183–228.

RHOADS, M. L., AND R. H. FETTERER. 1998. Purification and characterization of a secreted aminopeptidase for adult *Ascaris suum*. International Journal for Parasitology **28:** 1681–1690.

SCHULTZ, J., R. R. COPLEY, T. DOERKS, C. P. PONTING, AND P. BORK. 2000. SMART: a web based tool for the study of genetically mobile domains. Nucleic Acids Research **28:** 231–234.

THOMPSON, J. D., T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN, AND D. G. HIGGINS. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research **24:** 4876–4882.

WANG, C. C., AND R. L. STOTISH. 1978. Multiple leucine aminopeptidases in the oocysts of *Eimeria tenella* and their changes during sporulation. Comparative Biochemistry and Physiology **618:** 307–313.

WILLIAMS, R. B. 1998. Epidemiological aspects of the use of live anticoccidial vaccines for chickens. International Journal for Parasitology **28:** 1089–1098.